

SPECIFICATION  
NOVEL POLYPEPTIDES

TECHNICAL FIELD

The present invention relates to a fusion polypeptide comprising a polypeptide having a granulocyte colony stimulating factor (hereinafter referred to as "G-CSF") activity and a polypeptide having a platelet growth factor (thrombopoietin, hereinafter referred to as "TPO") activity, and DNA which codes for the fusion polypeptide. Since the fusion polypeptide of the present invention can form and amplify platelets and neutrophils simultaneously, it is useful for the treatment of anemia and the like.

BACKGROUND ART

Blood comprises hematopoietic cells such as erythrocytes, leukocytes, platelets and the like. These hematopoietic cells mature from only one kind of pluripotential blood stem cell through various differentiation steps. These steps undergo complex regulation by a group of proteinous factors which are generally referred to as cytokines. A certain type of cytokine takes part in the differentiation and multiplication of various hematopoietic cells. On the other hand, a certain type of hematopoietic cell undergoes regulation of its differentiation and multiplication by various types of cytokines. This is called overlapping cytokine actions. Among

these cytokine members, TPO and G-CSF are considered to have small overlapping actions.

TPO mainly takes part in the formation of platelets. Platelets are formed by the fragmentation of megakaryocytes, a hematopoietic cell which has<sup>b</sup> large nucleus and is present mainly in bone marrow. Platelets are essential for forming blood clots at damaged portions in blood vessels. Platelets also play important roles in not only blood coagulation but also injury healing by releasing proteins having other functions at the damaged portions. A significant decrease in the number of platelets may be fatal, because the body may easily bleed.

G-CSF is a cytokine which accelerates activation of neutrophils, a member of the leukocytes, and differentiation of neutrophils from their precursor cells. Neutrophils exert the first defense action when invaded by foreign enemies such as bacteria, viruses and the like. When the number of neutrophils is decreased, the body becomes defenseless against infection, and this too is also often fatal.

Current medical treatment of cancers often cause side effects in which pluripotential blood stem cells are damaged by the administration of a chemotherapeutic drug, irradiation of X-rays or bone marrow transplantation for the treatment of leukemia, thus decreasing the number of all hematopoietic cells. Apparently, it is markedly beneficial for thrombopenia and leukopenia patients to amplify the number of these cells by

the administration of cytokine, to suppress bleeding tendency and preventing infectious diseases.

A cytokine which can amplify platelets and neutrophils simultaneously has not been found, and there is no medicine having such an effect.

Leukemia inhibiting factors, stem cell factors, macrophage colony stimulating factors, granulocyte/macrophage colony stimulating factors, erythropoietin, interleukin (IL)-3, IL-6, IL-11, megakaryocyte colony stimulating factors and the like are known as substances which amplify platelets or enhance differentiation and multiplication of megakaryocytes [Metcalf *et al.*, *Blood*, 80, 50-56 (1990); Hunt *et al.*, *Blood*, 80, 904-911 (1992); Examined Japanese Patent Publication No. 6-11705; Hoffman *et al.*, *Blood Cells*, 13, 75-86 (1987); Mazur *et al.*, *Exp. Hematol.*, 15, 1123-1133 (1987); McNiece *et al.*, *Exp. Hematol.*, 16, 807-810 (1988); Lu *et al.*, *Brit. J. Hematol.*, 70, 149-156 (1988); Ishibashi *et al.*, *Proc. Natl. Acad. Sci. USA*, 86, 5953-5957 (1989); WO 95/21919; WO 95/18858]. It is understood that these many cytokine members amplify platelets by overlapping actions. Recently, it was revealed that a receptor ligand called c-mpl is a cytokine which has the highest activity among platelet amplifying factors and acts directly [de Sauvage *et al.*, *Nature*, 369, 533 (1994)].

As substances which multiply granulocytes, the above-mentioned IL-3, macrophage colony stimulating factors, granulocyte/macrophage colony stimulating factors and the like

are known, but G-CSF has the highest activity in terms of multiplying neutrophils selectively [Nicola et al., *J. Biol. Chem.*, 258, 9017 (1983)]. With regard to a polypeptide in which two different kinds of cytokine are fused, there are reports in Japanese Published Unexamined International Patent Application No. 500116/94, U.S. Patent 5,359,035, *Exp. Hematol.*, 21, 647-655 (1993) and *ibid.*, 18, 615 (1990) and the like.

However, nothing is known about a fusion polypeptide in which TPO is used as one of the fused cytokines.

An object of the present invention is to provide a fusion polypeptide which can produce and amplify platelets and neutrophils simultaneously. This fusion polypeptide allows the formation of megakaryocyte colonies and neutrophil colonies and the differentiation or maturation of megakaryocyte precursor and neutrophil precursor can be controlled.

#### DISCLOSURE OF THE INVENTION

The present invention relates to a fusion polypeptide which comprises a polypeptide having G-CSF activity and a polypeptide having TPO activity and DNA which codes for the fusion polypeptide. Also disclosed are fusion polypeptides in which a polypeptide having G-CSF activity and a polypeptide having TPO activity are fused via a spacer peptide and DNA which codes for the fusion polypeptide; and a polypeptide in which the fusion polypeptide comprising a polypeptide having G-

CSF activity and a polypeptide having TPO activity is chemically modified with a polyethylene glycol derivative. Also provided are anemia-treating compositions containing the fusion polypeptide as an active ingredient.

The fusion protein of the present invention has no mouse IL-3 activity.

As the polypeptide having G-CSF activity for use in the present invention, any protein may be used with the proviso that it has the requisite G-CSF activity, such as a polypeptide having the amino acid sequence shown in Table 1 [*Nature*, 319, 415 (1986)] .

Also useful is a protein which has an amino acid sequence derived from the amino acid sequence shown in Table 1 by substitution, deletion or addition of one or more amino acids, and examples thereof include hG-CSF derivatives shown in Table 2 and described in Japanese published Unexamined Patent Application No. 267299/88, Japanese Published Unexamined Patent application No. 299/88, and Japanese Published Unexamined International Patent Application No. 500636/88.

TABLE 1

X	Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser	Leu	Pro	Gln	Ser	Phe	Leu	Leu
1					5					10					15
Lys	Cys	Leu	Glu	Gln	Val	Arg	Lys	Ile	Gln	Gly	Asp	Gly	Ala	Ala	Leu
			20						25					30	
Gln	Glu	Lys	Leu	Cys	Ala	Thr	Tyr	Lys	Leu	Cys	His	Pro	Glu	Glu	Leu
			35					40					45		
Val	Leu	Leu	Gly	His	Ser	Leu	Gly	Ile	Pro	Trp	Ala	Pro	Leu	Ser	Ser
		50					55					60			
Cys	Pro	Ser	Gln	Ala	Leu	Gln	Leu	Ala	Gly	Cys	Leu	Ser	Gln	Leu	His
		65				70					75				
Ser	Gly	Leu	Phe	Leu	Tyr	Gln	Gly	Leu	Leu	Gln	Ala	Leu	Glu	Gly	Ile
		80				85				90					95
Ser	Pro	Glu	Leu	Gly	Pro	Thr	Leu	Asp	Thr	Leu	Gln	Leu	Asp	Val	Ala
			100						105					110	
Asp	Phe	Ala	Thr	Thr	Ile	Trp	Gln	Gln	Met	Glu	Glu	Leu	Gly	Met	Ala
			115					120					125		
Pro	Ala	Leu	Gln	Pro	Thr	Gln	Gly	Ala	Met	Pro	Ala	Phe	Ala	Ser	Ala
		130					135					140			
Phe	Gln	Arg	Arg	Ala	Gly	Gly	Val	Leu	Val	Ala	Ser	His	Leu	Gln	Ser
		145					150					155			
Phe	Leu	Glu	Val	Ser	Tyr	Arg	Val	Leu	Arg	His	Leu	Ala	Gln	Pro	
160						165				170				174	

(X represents H or Met.)

TABLE 2

Position from N-terminal amino acid (hG-CSF in Table 1)	Substituted amino acid in hG-CSF derivatives											
	a)	b)	c)	d)	e)	f)	g)	h)	i)	j)	k)	l)
1st (Thr)	*	Val	Cys	Tyr	Arg	*	Asn	Ile	Ser	*	Ala	*
3rd (Leu)	Glu	Ile	Ile	Ile	Thr	Thr	Glu	Thr	Thr	*	Thr	*
4th (Gly)	Lys	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Tyr	*
5th (Pro)	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	*	Arg	*
17th (Cys)	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser

\*: unsubstituted amino acid

As the polypeptide having TPO activity for use in the present invention, any protein may be used with the proviso that it has the requisite TPO activity, such as the c-mpl ligand which is a polypeptide having the amino acid sequence shown in Table 3 [*Nature*, 369, 533 (1994)], as well as leukemia inhibiting factors, stem cell factors, macrophage colony stimulating factors, granulocyte/macrophage colony stimulating factors, erythropoietin, interleukin (IL)-3, IL-6, IL-11, megakaryocyte colony stimulating factors and the like.



TABLE 3

Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Leu	Arg	Val	Leu	Ser	Lys	Leu
1			5						10					15
Leu	Arg	Asp	Ser	His	Val	Leu	His	Ser	Arg	Leu	Ser	Gln	Cys	Pro
			20						25					30
Glu	Val	His	Pro	Leu	Pro	Thr	Pro	Val	Leu	Leu	Pro	Ala	Val	Asp
			35						40					45
Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln	Met	Glu	Glu	Thr	Lys	Ala
			50						55					60
Gln	Asp	Ile	Leu	Gly	Ala	Val	Thr	Leu	Leu	Leu	Glu	Gly	Val	Met
			65						70					75
Ala	Ala	Arg	Gly	Gln	Leu	Gly	Pro	Thr	Cys	Leu	Ser	Ser	Leu	Leu
			80						85					90
Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	Gly	Ala	Leu	Gln
			95						100					105
Ser	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Pro	Gln	Gly	Arg	Thr	Thr	Ala
			110						115					120
His	Lys	Asp	Pro	Asn	Ala	Ile	Phe	Leu	Ser	Phe	Gln	His	Leu	Leu
			125						130					135
Arg	Gly	Lys	Val	Arg	Phe	Leu	Met	Leu	Val	Gly	Gly	Ser	Thr	Leu
			140						145					150
Cys	Val	Arg	Arg	Ala	Pro	Pro	Thr	Thr	Ala	Val	Pro	Ser	Arg	Thr
			155						160					165
Ser	Leu	Val	Leu	Thr	Leu	Asn	Glu	Leu	Pro	Asn	Arg	Thr	Ser	Gly
			170						175					180
Leu	Leu	Glu	Thr	Asn	Phe	Thr	Ala	Ser	Ala	Arg	Thr	Thr	Gly	Ser
			185						190					195
Gly	Leu	Leu	Lys	Trp	Gln	Gln	Gly	Phe	Arg	Ala	Lys	Ile	Pro	Gly
			200						205					210
Leu	Leu	Asn	Gln	Thr	Ser	Arg	Ser	Leu	Asp	Gln	Ile	Pro	Gly	Tyr
			215						220					225
Leu	Asn	Arg	Ile	His	Glu	Leu	Leu	Asn	Gly	Thr	Arg	Gly	Leu	Phe
			230						235					240
Pro	Gly	Pro	Ser	Arg	Arg	Thr	Leu	Gly	Ala	Pro	Asp	Ile	Ser	Ser
			245						250					255
Gly	Thr	Ser	Asp	Thr	Gly	Ser	Leu	Pro	Pro	Asn	Leu	Gln	Pro	Gly
			260						265					270
Tyr	Ser	Pro	Ser	Pro	Thr	His	Pro	Pro	Thr	Gly	Gln	Tyr	Thr	Leu
			275						280					285
Phe	Pro	Leu	Pro	Pro	Thr	Leu	Pro	Thr	Pro	Val	Val	Gln	Leu	His
			290						295					300
Pro	Leu	Leu	Pro	Asp	Pro	Ser	Ala	Pro	Thr	Pro	Thr	Pro	Thr	Ser
			305						310					315
Pro	Leu	Leu	Asn	Thr	Ser	Tyr	Thr	His	Ser	Gln	Asn	Leu	Ser	Gln
			320						325					330
Glu	Gly													
			332											

The polypeptide having G-CSF activity and the other polypeptide having TPO activity, which constitute the fused polypeptide of the present invention, are not particularly limited, provided that they contain respective activity-producing portions. For example, when the c-mpl ligand is used as the polypeptide having TPO activity, it may contain an amino acid sequence of the 153rd and 154th positions counting from the N-terminal amino acid.

Also included in the polypeptide of the present invention is a polypeptide in which a polypeptide having G-CSF activity and a polypeptide having TPO activity are fused via a spacer peptide. As the spacer peptide, any sequence may be used with the proviso that it does not spoil the G-CSF activity and TPO activity. For example, the peptide shown in Table 4 can be used as the spacer peptide.

TABLE 4

<u>Linker</u>
(GlyGlyGlySer) <sub>3</sub> Arg
(SerGlyGlyGly) <sub>4</sub> Arg
SerGlyGlyGlyArg
(SerGlyGlyGly) <sub>4</sub>
SerGlyGlyGly
(GlyGlyGlySer) <sub>3</sub>
(GlyGlyGlySer) <sub>2</sub>

Examples of the fusion polypeptide of the present invention include a polypeptide having the amino acid sequence shown in Sequence ID No. 1, 2 or 3 and a polypeptide derived from the amino acid sequence of the fusion polypeptide by addition, deletion or substitution of one or more amino acids within such a range that the G-CSF activity and TPO activity are not spoiled, having a homology of 40% or more with the amino acid sequence of the polypeptide. The homology is preferably 60% or more, and more preferably 80% or more.

The substitution, deletion or addition of amino acids can be carried out in accordance with known methods described for example in *Nucleic Acid Research*, 10, 6487 (1982); *Proc. Natl. Acad. Sci., USA*, 79, 6409 (1982); *Proc. Natl. Acad. Sci., USA*, 81, 5662 (1984); *Science*, 224, 1431 (1984); PCT WO 85/00817; *Nature*, 316, 601 (1985); *Gene*, 34, 315 (1985); *Nucleic Acid Research*, 13, 4431 (1985); and "Current Protocols in Molecular Biology", Chap. 8, Mutagenesis of Cloned DNA, John Wiley & Sons, Inc. (1989).

Also included in the fusion polypeptide of the present invention is a peptide having an amino acid sequence in which a secretion signal peptide is added to the N-terminal amino acid of the above-mentioned polypeptide; examples include a polypeptide having the amino acid sequence shown in Sequence ID

a  
NOS: 5, 7 or 9  
~~No. 4, 5 or 6...~~

In addition, a fusion polypeptide having G-CSF activity and TPO activity, in which at least one amino group of the

above-mentioned polypeptide is chemically modified with a polyalkylene glycol derivative, is also included in the fusion polypeptide of the present invention.

Examples of the polyalkylene derivative include a polyethylene glycol derivative, a polypropylene glycol derivative, a polyoxyethylene-polyoxypropylene copolymer derivative and the like. Polyethylene glycol-succinimidyl propionate is preferred.

The fusion polypeptide chemically modified with a polyethylene glycol derivative can be prepared in accordance with the method described in Japanese Examined Patent Publication No. 96558/95.

The DNA which codes for the fusion polypeptide (hereinafter referred to as "TPO-CSF") of the present invention can be obtained by polymerase chain reaction (PCR) and the like based on the known nucleotide sequences of a polypeptide having TPO activity and a polypeptide having G-CSF activity. It can also be obtained by chemical synthesis.

Examples of DNA which codes for TPO-CSF include a DNA containing a nucleotide sequence that codes for a polypeptide having the amino acid sequence shown in Sequence ID No. 1, 2 or 3 or a polypeptide derived from the amino acid sequence of the polypeptide by substitution, deletion or addition of one or more amino acids but having the G-CSF activity and TPO activity, such as a DNA which contains the nucleotide sequence shown in Sequence ID No. <sup>105: 4, 6 or 8</sup>~~4, 5 or 6~~.

Other examples are DNA's in which mutation such as substitution mutation, deletion mutation, insertion mutation or the like is introduced into the above-mentioned DNA within such a range that the G-CSF activity and TPO activity are not spoiled, which can be obtained, for example, by colony hybridization or plaque hybridization using a DNA containing the nucleotide sequence shown in Sequence ID, ~~No. 4, 5 or 6~~ <sup>NOS: 4, 6 or 8</sup> as a probe.

13 An example is a DNA which is identified by carrying out hybridization of a membrane filter on which colony- or plaque-originated DNA is fixed, at 65°C in the presence of 0.7 to 1.0 M sodium chloride using a DNA containing the nucleotide sequence shown in Sequence ID, ~~No. 4, 5 or 6~~ <sup>NOS: 4, 6 or 8</sup> as a probe, and subsequently washing the resulting filter at 65°C in 0.1 to 2-fold SSC solution (1-fold SSC contains 150 mM sodium chloride and 15 mM sodium citrate).

The hybridization techniques are described in "Molecular Cloning, A laboratory manual", second edition (edited by Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989).

All polypeptides encoded by the DNA defined in the foregoing are included in the TPO-CSF.

Examples of plasmids containing the TPO-CSF-encoding DNA include pBS-T153LND28, pBS-T154ND28 and pBS-T153ND28LN1. *Escherichia coli* TLN-1 as a colon bacillus containing pBS-T153LND28 and *Escherichia coli* TN-1 as a colon bacillus

containing pBS-T154ND28 have been deposited on February 16, 1995, in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Higashi 1-1-3, Tsukuba-shi, Ibaraki, Japan (the postal code: 305), and have been assigned the designations as FERM BP-5001 and FERM BP-5002, respectively.

In order to express the thus obtained TPO-CSF-encoding gene (hereinafter referred to as "TPO-CSF gene") in a host, a DNA fragment containing the TPO-CSF gene is first cleaved into a TPO-CSF gene-containing DNA of an appropriate length with restriction enzymes or DNA hydrolyzing enzymes and inserted into downstream site of a promoter gene on an expression vector and then the thus DNA-inserted expression vector is introduced into a host suitable for the expression vector.

As the host, any host capable of expressing the intended gene can be used. Examples thereof include microbial strains belonging to the genera *Escherichia*, *Serratia*, *Corynebacterium*, *Brevibacterium*, *Pseudomonas*, *Bacillus* and the like, as well as yeast strains, animal cell hosts and the like.

Useful as the expression vector is a vector which can replicate by itself in the above-mentioned host or can be inserted into its chromosome and has a promoter at a site where transcription of the TPO-CSF gene can be made.

When a microorganism such as *Escherichia coli* or the like is used as the host, it is desirable that the TPO-CSF expression vector can replicate by itself in the microorganism

and comprises a promoter, a ribosome binding sequence, the TPO-CSF gene and a transcription termination sequence. It may also contain a regulatory gene.

Examples of the expression vector include pBTrp2, pBTac1 and pBTac2 (all available from Boehringer-Mannheim Co.), pKYP10 (Japanese Published Unexamined Patent Application No. 110600/83), pKYP200 [*Agric. Biol. Chem.*, 48, 669 (1984)], pLSA1 [*Agric. Biol. Chem.*, 53, 277 (1989)], pGEL1 [*Proc. Natl. Acad. Sci., USA*, 82, 4306 (1985)], pBluescript (available from STRATAGENE Co.), pTrs30 [prepared from *Escherichia coli* JM109/pTrs30 (FERM BP-5407)], pTrs32 [prepared from *Escherichia coli* JM109/pTrs32 (FERM BP-5408)], pAGE107 [Japanese Published Unexamined Patent Application No. 22979/91; Miyaji et al., *Cytotechnology*, 3, 133 (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No. 227075/90) and pAMoERC3Sc CDM8 [Brian Seed et al., *Nature*, 329, 840 (1987)].

As the promoter, any one capable of exerting expression in a host such as *Escherichia coli* or the like can be used. Examples thereof include promoters originated from *Escherichia coli*, phages and the like, such as trp promoter (P<sub>trp</sub>), lac promoter (P<sub>lac</sub>), P<sub>L</sub> promoter, P<sub>R</sub> promoter and the like. Also useful are artificially designed and modified promoters such as a promoter prepared by connecting two P<sub>trp</sub> promoters in series (P<sub>trpx</sub> 2), tac promoter and the like.

As the ribosome binding sequence, any sequence capable of exerting expression in a host such as *Escherichia coli* or

the like can be used, but it is desirable to use a plasmid in which the ribosome binding sequence and the initiation codon are arranged with an appropriate distance (for example, 6 to 18 bases).

Any gene which codes for TPO-CSF can be used as the TPO-CSF gene, but it is desirable to use the gene by substituting its bases in such a manner that the DNA sequence of the gene has codons most suitable for its expression in host microorganisms.

Although the transcription termination sequence is not always necessary for the expression of the gene, it is desirable to arrange the transcription termination sequence preferably just downstream of the structural gene.

Examples of the host include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* DH5  $\alpha$ , *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Bacillus subtilis*, *Bacillus amyloliquefacience*, *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Brevibacterium flavum* ATCC 14067, *Brevibacterium lactofermentum* ATCC 13869, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniophilum* ATCC 15354 and the like.



When a yeast strain is used as the host, YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419) or the like may be used as the expression vector.

Any type of promoter can be used, provided that it can exert expression in yeast strain hosts. Examples thereof include promoters of genes of hexose kinase and the like glycolytic pathway enzymes, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MF  $\alpha$ 1 promoter, CUP 1 promoter and the like.

Examples of the host include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans*, *Schwanniomyces alluvius* and the like.

When animal cells are used as the host, examples of useful expression vectors include pcDNA I/Amp, pcDNA I, pcDM8 (all available from Funakoshi Co., Ltd.), pcDNA 3 (available from Invitrogen Co.), pAGE248, pAGE210 and the like.

Any promoter capable of exerting expression in the animal cell hosts can be used. For example, the promoter of human CMV IE (immediate early) gene may be used. Also, the enhancer of human CMV IE gene may be used together with the promoter.

Any gene which codes for TPO-CSF can be used as the TPO-CSF gene.

In general, only a portion of TPO-CSF expressed from the gene is secreted into the extracellular moiety, so that, in order to effect positive extracellular secretion of TPO-CSF

from the host, it is desirable to prepare and use a gene having a sequence in which a nucleotide sequence coding for a signal peptide is added to the gene, in accordance with the method of Paulson et al. [C. Paulson et al., *J. Biol. Chem.*, 264, 17619 (1989)] and the method of Lowe et al. [John. B. Lowe et al., *Proc. Natl. Acad. Sci., USA*, 86, 8227 (1989); John. B. Lowe et al., *Genes Develop.*, 4, 1288 (1990)].

As the host, namalwa cells, HBT5637 (Japanese Published Unexamined Patent Application No. 299/88), COS cells, CHO cells and the like may be used.

Introduction of TPO-CSF gene-containing DNA into animal cells can be effected by any method, provided that it can introduce DNA into animal cells. For example, an electroporation method [Miyaji et al., *Cytotechnology*, 3, 133 (1990)], a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), a lipofection method [Philip L. Felgner et al., *Proc. Natl. Acad. Sci., USA*, 84, 7413 (1987)] and the like may be used. Isolation and cultivation of a transformant can be effected in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90 or Japanese Published Unexamined Patent Application No. 257891/90.

TPO-CSF can be produced by cultivating the thus obtained transformant in accordance with the usually used cultivating method.

When a transformant obtained by using *Escherichia coli*, yeast or the like microorganism as the host is cultivated, the medium may be either a natural medium or a synthetic medium, with the proviso that it contains carbon sources, nitrogen sources, inorganic salts and the like which can be assimilated by the microorganism and cultivating of the transformant can be made efficiently.

As the carbon sources, those which can be assimilated by respective microorganisms are used, which include carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch, starch hydrolyzates and the like, organic acids such as acetic acid, propionic acid and the like and alcohols such as ethanol, propanol and the like.

Examples of useful nitrogen sources include ammonia, ammonium salts of various inorganic and organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate, ammonium phosphate and the like, and other nitrogen-containing compounds, as well as peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzate, soybean cake and soybean cake hydrolyzate, various fermented microbial cells and digests thereof.

Examples of useful inorganic materials include potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate and the like.

Cultivation is carried out under aerobic conditions by shaking, submerged-aerial stirring or the like. The temperature for the cultivation is preferably 15 to 40°C, and the period for the cultivation is generally 16 to 96 hours. The medium pH is controlled at 3.0 to 9.0 during the cultivation. Adjustment of the pH is carried out using an inorganic or organic acid, an alkaline solution, urea, calcium carbonate, ammonia and the like.

As occasion demands, antibiotics such as ampicillin, tetracycline and the like may be added to the medium during the cultivation.

When a microorganism transformed with an expression vector prepared using an inducible promoter is cultivated, an inducer may be added to the medium as occasion demands. For example, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) or the like may be added to the medium when a microorganism transformed with an expression vector prepared using lac promoter is cultivated, or indoleacetic acid (IAA) or the like when a microorganism transformed with an expression vector prepared using trp promoter is cultivated.

When a transformant obtained using animal cells as the host is cultivated, generally used RPMI 1640 medium, MEM medium (manufactured by Eagle Co. or GibcoBRL Co.), D-MEM medium (manufactured by GibcoBRL Co.) or any one of these media further supplemented with fetal bovine serum and the like may be used.

The cultivation is carried out, for example, in the presence of 5% CO<sub>2</sub>. The temperature for the cultivation is preferably 35 to 37°C, and the period for the cultivation is generally 3 to 7 days.

As occasion demands, antibiotics such as kanamycin, penicillin and the like may be added to the medium during the cultivation.

Productivity can be increased using a gene amplification system in which dihydrofolate reductase gene and the like are used, in accordance with the method described in Japanese Published Unexamined Patent Application No. 227075/90.

The TPO-CSF of the present invention obtained in this manner can be purified by commonly used protein purification techniques.

For example, when the TPO-CSF is not secreted into outside moiety of the host cells, a culture broth of the transformant is subjected to centrifugation to collect cells in the culture broth, and the thus collected cells are washed and then disrupted using a sonicator, French press, Manton Gaulin homogenizer, Dynomil or the like, thereby obtaining a cell-free extract. Thereafter, the cell-free extract is subjected to centrifugation, and the TPO-CSF is purified from the resulting supernatant fluid making use of various techniques including salting out with ammonium sulfate or the like salt, anion exchange chromatography on diethylaminoethyl (DEAE)-Sephadex or the like, hydrophobic chromatography on Butylsephadex,

Phenylsepharose or the like, molecular sieve-aided gel filtration and various types of electrophoresis such as isoelectric focusing and the like.

When the TPO-CSF is secreted, purified TPO-CSF can be obtained from a culture filtrate of the transformant in the same manner as the case of the above-mentioned treatment of cell-free extract supernatant.

When produced in *Escherichia coli* cells, it can be purified efficiently by the combination of the above-mentioned method with the method described in Japanese Published Unexamined Patent Application No. 267292/88.

Also, it is possible to produce the TPO-CSF of the present invention in the form of its fusion protein with another protein and to purify the product by affinity chromatography using a substance having affinity for the fused protein. For example, it is possible to produce the TPO-CSF of the present invention as its fusion protein with protein A and purify it by an immunoglobulin G-aided affinity chromatography, in accordance with the method of Lowe et al. [John. B. Lowe et al., *Proc. Natl. Acad. Sci., USA*, 86, 8227 (1989); John. B. Lowe et al., *Genes Develop.*, 4, 1288 (1990)].

In addition, it can also be purified by affinity chromatography using antibodies specific for a polypeptide which has G-CSF activity, such as antibodies specific for G-CSF.

The TPO-CSF of the present invention can be used as it is or as pharmaceutical compositions in various dosage forms.

The pharmaceutical compositions of the present invention are produced by mixing an effective amount of TPO-CSF as the active ingredient uniformly with pharmacologically acceptable carriers.

Preferably, these pharmaceutical compositions may be prepared in the form of unit dose packages suitable for injection.

Injections for use in injection administration can be prepared by using a carrier such as distilled water, a salt solution of sodium chloride or of a mixture of sodium chloride with other inorganic salts, a sugar solution of mannitol, lactose, dextran, glucose or the like, an amino acid solution of glycine, arginine or the like, an organic acid solution, an organic base solution or a mixture solution comprising a salt solution and a sugar solution. In that case, the composition can be made into solutions, suspensions or dispersions in the usual way using auxiliaries which include an osmotic pressure adjusting agent, a plant oil such as sesame oil or soybean oil and a surface active agent such as lecithin or a nonionic surface active agent. These solutions can be made into solid preparations by powder making, freeze drying and the like means, which are dissolved again prior to their use.

The above-mentioned pharmaceutical compositions which contain the TPO-CSF of the present invention as the active

ingredient are useful for the treatment anemia or patients who become anemic as a result of treatment of diseases.

#### BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 is an illustration showing construction of a plasmid containing DNA which codes for TPO-ND28 (1).

Fig. 2 is an illustration showing construction of a plasmid containing DNA which codes for TPO-ND28 (2).

Fig. 3 is an illustration showing construction of a plasmid containing DNA which codes for TPO-ND28 (3).

#### BEST MODE OF CARRYING OUT THE INVENTION

##### Example 1 Preparation of DNA which codes for TPO-CSF

A DNA which codes for TPO-CSF was prepared in the following manner, using a DNA which codes for a polypeptide ND28 in which the 1st position amino acid residue of the amino acid sequence of human G-CSF was substituted by alanine (Ala), and the 3rd position amino acid by threonine (Thr), the 4th position amino acid by tyrosine (Tyr), the 5th position amino acid by arginine (Arg) and the 17th position amino acid by serine (Ser) (Japanese Published Unexamined Patent Application No. 267292/88) as a DNA which codes for a polypeptide having G-CSF activity, and a DNA that codes for a polypeptide having the amino acid sequence of Table 3 (de Sauvage *et al.*, *Nature*, 369, 533 (1994); hereinafter referred to as "TPO") as a DNA which codes for a polypeptide having TPO activity. The fusion



polypeptide of TPO and ND28 is abbreviated as TPO-ND28 hereinafter.

#### 1. Preparation of TPO gene

A TPO-encoding gene (hereinafter referred to as "TPO gene") for use in the preparation of TPO-ND28 was obtained by PCR in the following manner on the basis of the nucleotide sequence reported by de Sauvage et al. [*Nature*, 369, 533 (1994)].

A DNA shown in Sequence ID No. 7 containing 5' end nucleotide sequence of the TPO gene (hereinafter referred to as "primer 1") and a DNA shown in Sequence ID No. 8 containing 3' end nucleotide sequence of the TPO gene (hereinafter referred to as "primer 2") were synthesized using 380A DNA synthesizer of Applied Biosystems, Inc. In order to facilitate the cloning, a restriction enzyme recognition sequence was added to the terminus of each primer.

Amplification and cloning of the TPO gene translation region sequence were carried out by reverse transcription PCR using the primers 1 and 2, human liver poly A<sup>+</sup> mRNA (manufactured by Clontech Co., product No. CL 6510-1) mRNA and SuperScript Preamplification System for First Strand cDNA Synthesis Kit (manufactured by GibcoBRL Co.).

A 0.013 ml portion of aqueous solution containing 1,000 ng of human liver poly A<sup>+</sup> mRNA and 500 ng of oligo(dt) 12-18 (included in the kit) was treated at 70°C for 10 minutes and then allowed to stand in ice for 1 minute.

The resulting solution was mixed with 0.002 ml of ten times-concentrated synthesis buffer, 0.001 ml of 10 mM dNTP mix, 0.002 ml of 0.1 M DTT and 0.001 ml of SuperScript II RT (200 kU/ml) (all included in the kit), and the mixture was allowed to stand at room temperature for 10 minutes and then incubated at 42°C for 50 minutes. After completion of the incubation, the mixture was heated at 90°C for 5 minutes to terminate the reverse transcription reaction.

The reaction solution was mixed with 0.001 ml of E. coli RNase H (2,000 U/ml; included in the kit) and incubated at 37°C for 20 minutes.

A 0.1 ml portion of a reaction solution containing 0.005 ml of the above reaction solution, 400 nM of the primer 1, 400 nM of the primer 2, 20 mM of Tris-HCl (pH 8.2), 10 mM of potassium chloride, 0.01 mg/ml of bovine serum albumin (hereinafter referred to as "BSA"), 2 mM of magnesium chloride, 6 mM of ammonium sulfate, 0.1% Triton X-100, 10% dimethyl sulfoxide (hereinafter referred to as "DMSO"), 0.05 mM of deoxyadenosine triphosphate (hereinafter referred to as "dATP"), 0.05 mM of deoxycytidine triphosphate (hereinafter referred to as "dCTP"), 0.05 mM of deoxyguanosine triphosphate (hereinafter referred to as "dGTP") and 0.05 mM of deoxythymidine triphosphate (hereinafter referred to as "dTTP") was mixed with 2.5 units of Pfu polymerase (manufactured by Stratagene Co.) to carry out PCR using PERKIN ELMER CETUS DNA Thermal Cycler (manufactured by Takara Shuzo Co., Ltd.) by 35

time repetition of a three step incubation at 94°C for 45 seconds, at 50°C for 1 minute and at 72°C for 2 minutes.

The resulting reaction solution was subjected to phenol/chloroform extraction and ethanol precipitation, and the thus obtained precipitate was dissolved in 0.015 ml of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (hereinafter referred to as "EDTA")].

The thus prepared solution was mixed with restriction enzymes HindIII and KpnI to cleave the DNA amplified by PCR.

The resulting solution was subjected to an agarose gel electrophoresis, and a HindIII-KpnI treated DNA of about 1.1 kb was isolated from the agarose gel.

Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the thus isolated DNA (50 ng) was ligated with a HindIII-KpnI cleaved 2.9 kb fragment (30 ng) of a plasmid vector pBlueScript II SK(-) having a multicloning site (manufactured by Stratagene Co.) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, an *Escherichia coli* strain DH5 $\alpha$  (Library Efficiency DH5 $\alpha$  Competent Cell, manufactured by GibcoBRL Co.) was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method [Birnboim *et al.*, *Nucleic Acids Res.*, 7, 1513 (1979)].

Nucleotide sequence of the insertion fragment in each plasmid was determined using Taq DyeDeoxy Terminator Cycle Sequencing Kit (manufactured by Applied Biosystems Japan Inc., product No. 401113) and ABI373A DNA Sequencer (manufactured by Applied Biosystems Japan Inc.). In determining the nucleotide sequence, six DNA's having the nucleotide sequences of Sequence ID Nos. 9 to 13 or 14 and two primers having the nucleotide sequence shown in Sequence ID No. 15 or 16 containing a nucleotide sequence in the vector were synthesized based on the nucleotide sequence of TPO gene [de Sauvage *et al.*, *Nature*, 369, 533 (1994)] and used as primers for the nucleotide sequence determination.

Determination of nucleotide sequence was carried out in accordance with the instructions attached to the kit and apparatus.

Of the above-mentioned plasmids, a plasmid pBS-TPO332 which coincided with the reported nucleotide sequence of the insertion fragment of TPO gene was used in the subsequent procedures.

## 2. Construction and expression of DNA which codes for TPO-ND28

Using the TPO-encoding DNA obtained in Example 1-1 and the ND28-encoding DNA obtained by the method described in Japanese Published Unexamined Patent Application No. 267292/88,

a fusion polypeptide of TPO and ND28 (TPO on the N-terminal side and ND28 on the C-terminal side), TPO-ND-28, was prepared in the following manner.

1) Construction of DNA (Sequence ID No. 5) which codes for TPO-ND28 (1) [Sequence ID No. 2; a type constructed through a linker (Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Arg; sequence ID No. 17)]

Though the mature type TPO comprises 332 amino acids, it is reported that its shortened protein consisting of its N-terminal side 153 amino acids can show the same activity of the complete length TPO [de Sauvage et al., *Nature*, 369, 533 (1994)], so that a DNA which codes for TPO-ND28 (1) in which the 153 amino acids from the N-terminal of TPO, used as its N-terminal side, was fused with the complete length ND28 (174 amino acids) as the C-terminal side through a linker (Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Arg) was prepared in the following manner (cf. Fig. 1).

(i) Preparation of DNA which codes for the TPO moiety of TPO-ND28 (1)

In order to prepare a DNA which codes for the TPO moiety of TPO-ND28 (1) by means of PCR, a DNA primer having a nucleotide sequence (Sequence ID No. 18) which corresponds to the linker was synthesized as the 3' end primer (hereinafter referred to as "primer 3").

Using the thus synthesized primer 3 and the primer 1 and pBS-TPO332, PCR was carried out in the following manner.

A 0.1 ml portion of a reaction solution containing 10 ng of pBS-TPO332, 400 nM of the primer 3, 400 nM of the primer 1, 20 mM of Tris-HCl (pH 8.2), 10 mM of potassium chloride, 0.01 mg/ml of BSA, 2 mM of magnesium chloride, 6 mM of ammonium sulfate, 0.1% Triton X-100, 10% DMSO, 0.05 mM of dATP, 0.05 mM of dCTP, 0.05 mM of dGTP and 0.05 mM of dTTP was mixed with 2.5 units of Pfu polymerase to carry out PCR using PERKIN ELMER CETUS DNA Thermal Cyclor (manufactured by Takara Shuzo Co., Ltd.) by 18 time repetition of a three step incubation at 94°C for 45 seconds, at 50°C for 1 minute and at 72°C for 1 minute.

The resulting reaction solution was subjected to phenol/chloroform extraction and ethanol precipitation, and the thus obtained precipitate was dissolved in 0.015 ml of TE buffer.

The thus prepared solution was mixed with restriction enzymes HindIII and XbaI to cleave the DNA amplified by PCR.

The resulting solution was subjected to an agarose gel electrophoresis, and a HindIII-XbaI treated DNA fragment of about 0.6 kb was isolated from the agarose gel.

Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the thus isolated DNA fragment (100 ng) was ligated with a HindIII-XbaI cleaved 2.9 kb fragment (50 ng) of pBlueScript II SK(-) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting

transformant was spread on LB agar medium containing 50 µg/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Nucleotide sequence of the insertion fragment in each plasmid was determined using Taq DyeDeoxy Terminator Cycle Sequencing Kit and ABI373A DNA Sequencer (manufactured by Applied Biosystems Japan Inc.). In determining the nucleotide sequence, primers having the nucleotide sequences of Sequence ID Nos. 9 to 12, 15 and 16 were used as primers for the nucleotide sequence determination.

Determination of nucleotide sequence was carried out in accordance with the instructions attached to the kit and apparatus.

Of the above-mentioned plasmids, plasmid pBS-T153LND which coincided with the reported nucleotide sequence of the insertion fragment of TPO gene was used in the subsequent procedures.

(ii) Preparation of DNA which codes for the ND28 moiety of TPO-ND28 (1)

In order to prepare a DNA which codes for the ND28 moiety of TPO-ND28 (1) by means of PCR, a primer having a nucleotide sequence (Sequence ID No. 19) which corresponds to the linker and the amino acid sequence of ND28 was synthesized as the 5' end primer (hereinafter referred to as "primer 4"), and a primer having a nucleotide sequence (Sequence ID No. 20)

which corresponds to the C-terminal side amino acid sequence of ND28 was synthesized as the 3' end primer (hereinafter referred to as "primer 5").

Using the thus synthesized primers and plasmid pCfBD28 (Japanese Published Unexamined Patent Application No. 267292/88), PCR was carried out in the following manner.

A 0.1 ml portion of a reaction solution containing 10 ng of pCfBD28, 400 nM of the primer 4, 400 nM of the primer 5, 20 mM of Tris-HCl (pH 8.2), 10 mM of potassium chloride, 0.01 mg/ml of BSA, 2 mM of magnesium chloride, 6 mM of ammonium sulfate, 0.1% Triton X-100, 10% DMSO, 0.05 mM of dATP, 0.05 mM of dCTP, 0.05 mM of dGTP and 0.05 mM of dTTP was mixed with 2.5 units of Pfu polymerase to carry out PCR using PERKIN ELMER CETUS DNA Thermal Cycler (manufactured by Takara Shuzo Co., Ltd.) by 18 time repetition of a three step incubation at 94°C for 45 seconds, at 50°C for 1 minute and at 72°C for 1 minute.

The resulting reaction solution was subjected to phenol/chloroform extraction and ethanol precipitation, and the thus obtained precipitate was dissolved in 0.015 ml of TE buffer.

The thus prepared solution was mixed with restriction enzymes SacII and XbaI to cleave the DNA amplified by PCR.

The resulting solution was subjected to an agarose gel electrophoresis, and a SacII-XbaI cleaved DNA fragment of about 0.5 kb was isolated from the agarose gel.



Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the thus isolated DNA fragment (100 ng) was ligated with a SacII-XbaI cleaved 2.9 kb fragment (50 ng) of pBlueScript II SK(-) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Nucleotide sequence of the insertion fragment in each plasmid was determined using Taq DyeDeoxy Terminator Cycle Sequencing Kit and ABI373A DNA Sequencer. In determining the nucleotide sequence, two DNA's having the nucleotide sequence of Sequence ID No. 21 or 22 containing a nucleotide sequence of the ND28-encoding DNA and two DNA's having the nucleotide sequence of Sequence ID No. 15 or 16 containing a sequence present in the vector were used as primers for the nucleotide sequence determination.

Determination of nucleotide sequence was carried out in accordance with the instructions attached to the kit and apparatus.

Of the above-mentioned plasmids, plasmid pBS-LND28 in which the nucleotide sequence of the insertion fragment

coincided with the nucleotide sequences of the ND28 gene and primers was used in the subsequent procedures.

(iii) Preparation of DNA which codes for TPO-ND28 (1)

The DNA's respectively which code for the TPO moiety and ND28 moiety prepared in Example 1-2-1)-(i) and (ii) were fused in the following manner.

A 2,000 ng portion of pBS-T153LND was cleaved with restriction enzymes SacII and XbaI and subjected to an agarose gel electrophoresis to isolate a DNA fragment of about 3.5 kb.

Also, a 500 ng portion of pBS-LND28 was cleaved with restriction enzymes SacII and XbaI and subjected to an agarose gel electrophoresis to isolate a DNA fragment of about 0.5 kb.

Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the DNA fragment of about 3.5 kb (100 ng) was ligated with the DNA fragment of about 0.5 kb (100 ng) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Structures of these plasmids were examined using restriction enzymes SacII and XbaI, and plasmid pBS-T153LND28 having a structure in which both of the DNA fragments are ligated with each other was used in the subsequent procedures.

2) Construction of DNA (Sequence ID No. 4) which codes for TPO-ND28 (2) [Sequence ID No. 1; a type constructed without a linker]

A DNA which codes for TPO-ND28 (2) in which the 154 amino acids of TPO from its N-terminal were fused with the N-terminal of D28 (174 amino acids) was prepared in the following manner (cf. Fig. 2).

(i) Preparation of DNA which codes for the TPO moiety of TPO-ND28 (2)

In order to prepare a DNA which codes for the TPO moiety of TPO-ND28 (2) by means of PCR, a primer having a nucleotide sequence shown in Sequence ID No. 23 which has a nucleotide sequence that corresponds to the amino acid sequences of TPO and ND28 was synthesized as the 3' side primer (hereinafter referred to as "primer 6").

Using the thus synthesized primer 6 and the primer 1 and pBS-TPO332, PCR was carried out in the following manner.

A 0.1 ml portion of a reaction solution containing 10 ng of pBS-TPO332, 400 nM of the primer 1, 400 nM of the primer 6, 20 mM of Tris-HCl (pH 8.2), 10 mM of potassium chloride, 0.01 mg/ml of BSA, 2 mM of magnesium chloride, 6 mM of ammonium sulfate, 0.1% Triton X-100, 10% DMSO, 0.05 mM of dATP, 0.05 mM of dCTP, 0.05 mM of dGTP and 0.05 mM of dTTP was mixed with 2.5 units of Pfu polymerase to carry out PCR using PERKIN ELMER CETUS DNA Thermal Cyclor by 18 time repetition of a three step

incubation at 94°C for 45 seconds, at 50°C for 1 minute and at 72°C for 1 minute.

The resulting reaction solution was subjected to phenol/chloroform extraction and ethanol precipitation, and the thus obtained precipitate was dissolved in 0.015 ml of TE buffer.

The thus prepared solution was mixed with restriction enzymes HindIII and XhoI to cleave the DNA amplified by PCR.

The resulting solution was subjected to an agarose gel electrophoresis, and a HindIII-XhoI cleaved DNA fragment of about 0.5 kb was isolated from the agarose gel.

Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the thus isolated DNA fragment (100 ng) was ligated with a HindIII-XhoI cleaved 2.9 kb fragment (50 ng) of pBlueScript II SK(-) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Nucleotide sequence of the insertion fragment in each plasmid was determined using Taq DyeDeoxy Terminator Cycle Sequencing Kit and ABI373A DNA Sequencer (manufactured by Applied Biosystems Japan Inc.). In determining the nucleotide

sequence, primers having the nucleotide sequences of Sequence ID Nos. 9 to 12, 15 and 16 were used as primers for the nucleotide sequence determination.

Determination of nucleotide sequence was carried out in accordance with the instructions attached to the kit and apparatus.

Of the above-mentioned plasmids, plasmid pBS-T154ND in which the nucleotide sequence of the insertion fragment coincided with the nucleotide sequences of the TPO gene and primers was used in the subsequent procedures.

(ii) Preparation of DNA which codes for TPO-ND28 (2)

The DNA which codes for the TPO moiety prepared in Example 1-2-2)-(i) and the DNA which codes for the ND28 moiety prepared in Example 1-2-1)-(ii) were fused in the following manner.

A 200 ng portion of pBS-T154ND was cleaved with restriction enzymes KpnI and XhoI and subjected to agarose gel electrophoresis to isolate a DNA fragment of about 3.5 kb.

Also, a 500 ng portion of pBS-LND28 was cleaved with restriction enzymes KpnI and XhoI and subjected to agarose gel electrophoresis to isolate a DNA fragment of about 0.5 kb.

Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the DNA fragment of about 3.5 kb (100 ng) was ligated with the DNA fragment of about 0.5 kb (100 ng) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Structures of these plasmids were examined using restriction enzymes KpnII and XhoI, and plasmid pBS-T154ND28 having a structure in which both of the DNA fragments are ligated with each other was used in the subsequent procedures.

3) Construction of DNA (Sequence ID No. 6) which codes for TPO-ND28 (3) [Sequence ID No. 3; a type constructed through a linker (Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Arg; sequence ID No. 24)]

A DNA which codes for TPO-ND28 (3) in which the 153 amino acids from the N-terminal of TPO, used as its N-terminal side, was fused with the complete length ND28 (174 amino acids) as the C-terminal side through a linker (Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Arg) was prepared in the following manner (cf. Fig. 3).

In order to ligate the DNA which codes for the TPO moiety prepared in Example 1-2-2)-(i) with the DNA which codes for the ND28 moiety prepared in Example 1-2-1)-(ii) through a linker (Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Arg), two DNA's shown in Sequence ID Nos. 25 and 26 having nucleotide sequences which form SplI-BbeI complementary

termini on both sides corresponding to the amino acid sequences of linkers were synthesized.

A 0.02 ml portion of a solution containing 0.01 mM of the DNA shown in Sequence ID No. 25, 5 mM of ATP, 50 mM of Tris-HCl (pH 8.0), 10 mM of magnesium chloride and 5 mM of dithiothreitol was mixed with 10 units of T4 Polynucleotide Kinase (manufactured by Takara Shuzo Co., Ltd.), and the mixture was allowed to stand at 37°C for 30 minutes and then heated at 70°C for 3 minutes to obtain treating solution (1).

The DNA shown in Sequence ID No. 26 was also treated in the same manner to obtain treating solution (2).

Treating solution (1) was mixed with treating solution (2), and the mixture was incubated at 90°C for 5 minutes and then gradually cooled to 22°C spending 3 hours to prepare double-stranded DNA.

The thus prepared double-stranded DNA was inserted into the connecting site of the TPO-coding gene and ND28-coding gene of pBS-T154ND28 obtained in Example 1-2-2)-(ii) in the following manner.

A 2,000 ng portion of pBS-T154ND28 was cleaved with restriction enzymes BbeI and SplI and subjected to an agarose gel electrophoresis to isolate a DNA fragment of about 4.0 kb.

Using DNA Ligation Kit Ver. 1 (manufactured by Takara Shuzo Co., Ltd.), the DNA fragment of about 4.0 kb (100 ng) was ligated with the above-mentioned double-stranded DNA (12.5 pmole) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Nucleotide sequence of the insertion fragment in each plasmid was determined using Taq DyeDeoxy Terminator Cycle Sequencing Kit and ABI373A DNA Sequencer. In determining the nucleotide sequence, two DNA's shown in Sequence ID Nos. 12 and 22 were used as primers. Determination of nucleotide sequence was carried out in accordance with the instructions attached to the kit and apparatus.

Of these plasmids, plasmid named pBS-T153ND28LN1 in which the nucleotide sequence of the insertion fragment coincided with the nucleotide sequence of the linker DNA was used in the subsequent procedures.

## Example 2 Production of TPO-CSF

The TPO-CSF was produced by effecting expression of the DNA which codes for the TPO-CSF in animal cells in the following manner.

### 1) Production of TPO-ND28 (1) and TPO-ND28 (2)

Plasmid pcDNA3 (manufactured by Invitrogen Co.) was cleaved with EcoRI and NotI and subjected to an agarose gel



electrophoresis to isolate a DNA fragment (vector side) of about 5.4 kb.

Also, pBS-T153LND28 and pBS-T154ND28 obtained in Example 1-2-1)-(iii) and Example 1-2-2)-(ii) were separately cleaved with EcoRI and NotI and subjected to agarose gel electrophoresis to isolate a DNA fragment (insert side) of about 1.1 kb from each plasmid.

Using DNA Ligation Kit Ver. 1, the vector side DNA fragment of about 5.4 kb (100 ng) was ligated with each of the insert side DNA fragments (100 ng) (volume of the reaction solution: 0.018 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Structure of each plasmid was examined using restriction enzymes EcoRI and NotI to select plasmids containing respective inserts having a structure in which the vector side and insert side DNA fragments are ligated with each other, and plasmid pCD-153LND28 containing a TPO-ND28 (1) encoding gene and plasmid pCD-154ND28 containing a TPO-ND28 (2) encoding gene were used in the subsequent procedure.

Plasmid pCD-153LND28 or pCD-154ND28 was introduced into animal cells by electroporation [Potter et al., Proc. Natl.

Acad. Sci., USA, 81, 7161 (1984)] and its expression was effected in the following manner.

COS 7 cells were cultivated in D-MEM medium (manufactured by GibcoBRL Co., product No. 11885-50) which was further supplemented with 10% fetal bovine serum.

The COS 7 cells obtained by cultivation were suspended in K-PBS buffer (137 mM potassium chloride, 2.7 mM sodium chloride, 8.1 mM disodium hydrogenphosphate, 1.5 mM sodium dihydrogenphosphate, 4 mM magnesium chloride) to prepare a cell suspension of  $8 \times 10^8$  cells/ml.

A 0.2 ml portion of the cell suspension was injected into a Pulser Cuvette (manufactured by BIO RAD LABORATORIES) having a slit width of 0.2 cm.

A 4  $\mu$ g portion of pCD-153LND28 or pCD-154ND28 was added to the cuvette, thoroughly mixed with the suspension and then subjected to pulse application using an electroporation apparatus (Gene Pulser, manufactured by BIO RAD LABORATORIES) under conditions of 200  $\Omega$ , 0.3 kv/cm and 0.125 mF.

The pulse-treated solution was allowed to stand in ice for 5 minutes, suspended in 10 ml of D-MEM medium supplemented with 10% fetal bovine serum and then cultivated at 37°C for 72 hours in a CO<sub>2</sub> incubator.

The culture broth was subjected to centrifugation, and the resulting culture supernatant was filtered through a filter of 220 nm pore size to obtain a solution of TPO-ND28 (1) or TPO-ND28 (2).

## 2) Production of TPO-ND28 (3)

A plasmid PAGE210 was used as the vector for use in the expression of TPO-ND28 (3). The vector PAGE210 is a derivative of PAGE248 [Sasaki et al., *J. Biol. Chem.*, 269, 14730, (1994)], in which the Moloney murine leukemia virus promoter (XhoI-HindIII fragment) has been replaced by SV40 early promoter (XhoI-HindIII fragment) of PAGE103 [Mizukami et al., *J. Biochem.*, 101, 1307 (1987)].

Plasmid PAGE210 was cleaved with KpnI and HindIII and subjected to an agarose gel electrophoresis to isolate a DNA fragment (vector side) of about 9.0 kb.

Separately from this, pBS-TPO322 obtained in Example 1-1 was cleaved with KpnI and HindIII, and pBS-153ND28LN1 obtained in Example 1-2-3) was cleaved with KpnI and then partially with HindIII, and each of the resulting cleaved fragments was subjected to an agarose gel electrophoresis to isolate a DNA fragment (insert side) of about 1.1 kb from each plasmid.

Using DNA Ligation Kit Ver. 1, the vector side DNA fragment of about 9.0 kb (100 ng) was ligated with each of the insert side DNA fragments of about 1.1 kb (100 ng) (volume of the reaction solution: 0.012 ml).

Using this reaction solution, the *Escherichia coli* strain DH5 $\alpha$  was transformed in the usual way, and the resulting transformant was spread on LB agar medium containing 50  $\mu$ g/ml of ampicillin and cultivated overnight at 37°C.

Plasmids were isolated from several transformant strains grown on the medium in accordance with a known method.

Structure of each plasmid was examined using a restriction enzyme KpnI to select plasmids containing respective inserts having a structure in which the vector side and insert side DNA fragments are ligated with each other, and plasmid pAGE210-T332 containing TPO encoding gene and plasmid pAGE210-LN1 containing TPO-ND28 (3) encoding gene were used in the subsequent procedure.

Plasmid pAGE210-T332 or pAGE210-LN1 was introduced into animal cells by electroporation.

CHO cells were cultivated in MEM medium (1) (manufactured by GibcoBRL Co., product No. 19000-024) which was further supplemented with 10% fetal bovine serum.

The CHO cells obtained by cultivation were suspended in K-PBS buffer to prepare a cell suspension of  $8 \times 10^6$  cells/ml.

A 0.2 ml portion of the cell suspension was injected into Pulser Cuvette having a slit width of 0.2 cm.

A 4  $\mu$ g portion of pAGE210-T332 or pAGE210-LN1 was added to the cuvette, thoroughly mixed with the suspension and then subjected to pulse application using an electroporation apparatus, Gene Pulser, under conditions of 0.35 kv/cm and 0.25 mF.

The pulse-treated solution was allowed to stand in ice for 5 minutes, suspended in 10 ml of MEM medium supplemented

with 10% fetal bovine serum and then cultivated at 37°C for 24 hours in a CO<sub>2</sub> incubator.

The thus cultivated cells were again cultivated for 2 weeks in MEM medium (1) supplemented with 10% fetal bovine serum and 0.3 mg/ml of hygromycin.

The resulting cells were further cultivated for 2 weeks in MEM medium (2) (manufactured by GibcoBRL Co., code No. 12000-022) supplemented with 10% fetal bovine serum and 50 nM methotrexate (hereinafter referred to as MTX).

The cultivation was repeated in the same manner by successively increasing the MTX concentration to 100 nM, 500 nM and 1,000 nM in that order, thereby obtaining strains resistant to 1,000 nM MTX.

Each of the 1,000 nM MTX resistant strains was grown in MEM medium (2) supplemented with 10% fetal bovine serum, the medium was exchanged with a serum-free medium for CHO cell use, CHO-S-SFMII (manufactured by GibcoBRL Co., code No. 12052-015), and then the strain was cultivated again for 96 to 144 hours.

By subjecting the culture broth to centrifugation, a culture supernatant containing TPO or TPO-ND28 (3) was obtained.

### Example 3 Purification of TPO-ND28 (3) and TPO

A 1,000 ml portion of TPO-ND28 (3) or TPO obtained in Example 2-2) was concentrated to 50 ml using Centriprep

(manufactured by Amicon Co.) to prepare a concentrated solution.

A 50 ml portion of each of the concentrated solutions was applied to XK50 column (manufactured by Pharmacia K.K.) which has been packed with 1,000 ml of Sephacryl S-200 resin (manufactured by Pharmacia K.K.) and filled with a phosphate buffer (9.4 mM sodium phosphate (pH 7.2), 137 mM NaCl, 2.7 mM KCl).

Elution of TPO-ND28 (3) or TPO was effected by passing the phosphate buffer through the column at a flow rate of 3 ml/minute.

The eluates were pooled for every 12.5 minutes, and the resulting fractions were checked for their TPO and G-CSF activities by an MTT assay method which will be described later, thereby obtaining purified TPO-ND28 (3) or TPO.

#### Example 4    Modification of TPO-ND28 (3) with polyethylene glycol

To ice-cooled water was added 20 kd PEG-succinimidyl propionate (manufactured by Shearwater Polymers Co.) to a final concentration of 400 mg/ml.

A 50 µl portion of the thus prepared aqueous solution was mixed with 200 µl of the TPO-ND28 (3) solution obtained in Example 3 and 150 µl of distilled water. The mixture was allowed to stand for 12 hours at 4°C, thereby effecting modification of TPO-ND28 (3) by polyethylene glycol.

The TPO-ND28 (3) thus modified with polyethylene glycol (hereinafter referred to as PEG-TPO-ND28 (3)) was applied to a column of Super Rose 610/30 (manufactured by Pharmacia K.K.) which has been filled in advance with a phosphate buffer (9.4 mM sodium phosphate (pH 7.2), 137 mM NaCl, 2.7 mM KCl).

Elution was effected by passing the phosphate buffer through the column at a flow rate of 0.5 ml/minute.

The eluates were pooled for every 1 minute, and the resulting fractions were checked for their G-CSF and TPO activities by MTT assay method which will be described later.

The results are shown in Table 5.

The G-CSF and TPO activities originated from unmodified TPO-ND28 (3) were detected 34 to 40 minutes after commencement of the elution, and the G-CSF and TPO activities originated from PEG-TPO-ND28 (3) were detected after 16 to 28 minutes of the elution.

These results confirmed that polyethylene glycol-modified TPO-CSF having both G-CSF and TPO activities can be obtained.

TABLE 5

Elution time (minutes)	0	10	14	16	18	20	22	24	26	28	30	32	34	36	38	40
G-CSF activity	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+
TPO activity	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	+

-: no activity; +: activity

#### Test Example 1 Measurement of TPO-ND28 molecular weight

Using the TPO-ND28 (1) solution obtained in Example 2-1), its molecular weight was measured by a gel filtration chromatography in the following manner.

A 0.2 ml portion of the TPO-ND28 (1) solution was applied to a column of Super Rose 610/30 (manufactured by Pharmacia K.K.) which has been equilibrated in advance with a phosphate buffer (9.4 mM sodium phosphate (pH 7.2), 137 mM NaCl, 2.7 mM KCl), and elution of TPO-ND28 (1) was effected by passing the phosphate buffer through the column at a flow rate of 0.5 ml/minute.

The eluates were pooled for every 0.5 minute, and the resulting fractions were checked for their TPO and G-CSF activities by an MTT assay method which will be described later.

Table 6 shows elution time from Super Rose and measured values of TPO and G-CSF activities.

The TPO and G-CSF activities reached the maximum after 33.5 minutes of the elution.



Separately from this, thyroglobulin (molecular weight: 670,000), aldolase (molecular weight: 160,000), bovine serum albumin (molecular weight: 69,000) and G-CSF (molecular weight: 20,000) were used as the standard molecular weight proteins and passed through Super Rose to obtain relationship between elution time and molecular weight.

Molecular weight of TPO-ND28 (1) deduced from the 33.5 minutes of elution time was about 40,000.

TABLE 6

Elution time	0	20	30	32	33	33.5	34	35	37	42
TPO activity (A <sub>540</sub> )	0.08	0.07	0.08	0.19	0.30	0.32	0.29	0.18	0.09	0.08
G-CSF activity (A <sub>540</sub> )	0.00	0.00	0.03	0.14	0.29	0.32	0.30	0.22	0.05	0.00

### Test Example 2 Biological activity of TPO-CSF

Basic construction for the measurement of the cell growth-stimulating activity of a solution to be tested (TPO-ND28 solution) upon cells to be tested is as follows.

Each solution to be tested (TPO-ND28 solution), TPO standard solution and ND28 standard solution is made into 10-fold serial dilutions, and a 0.01 ml portion of each of the dilutions is added to each well of a microtiter plate.

Actively growing cells to be tested are collected from a culture broth by centrifugation, washed and then re-suspended

in a medium for testing use to a most suitable cell density for each testing.

The thus prepared cell suspension is dispensed in 0.09 ml portions into wells of the above-mentioned microtiter plate which has been prepared by dispensing dilutions of the solution to be tested, TPO standard solution or ND28 standard solution in 0.01 ml portions.

The microtiter plate is incubated at 37°C in a completely moist 5% CO<sub>2</sub> incubator and then used in the following testing.

A 0.01 ml portion of 0.5 mg/ml solution of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide] is added to each well, incubated for 4 hours, mixed with 0.15 ml of 0.1 N hydrochloric acid/isopropyl alcohol solution and then stirred to extract pigment from the cells, subsequently judging growth of the cells by measuring the amount of the pigment by its absorbance at 540 nm.

This method for the measurement of cell growth-stimulating activity is hereinafter called the MTT assay.

(1) Measurement of cell growth-stimulating activity upon Ba/F3 cells

The Ba/F3 cells which grow depending on the presence of mouse IL-3 were cultivated in Iscove's modified Dulbecco medium (hereinafter referred to as "IMDM") which has been supplemented with 10% heat-inactivated fetal calf serum (hereinafter

referred to as "FCS") and mouse IL-3 (culture supernatant of WEHI-3B).

Using the thus cultivated Ba/F3 cells, the cell growth-stimulating activity was measured by the MTT assay using the just described medium but in the absence of mouse IL-3.

The MTT assay was carried out with an inoculation density of 10,000 cells per well and by incubating the plate in 5% CO<sub>2</sub> for 48 hours.

Results of the MTT assay showed that each of TPO, ND28 and TPO-ND28 (1), (2) and (3) had no Ba/F3 cell growth-stimulating activity.

(2) Measurement of cell growth-stimulating activity upon Ba/F3-cmpl

The Ba/F3-cmpl cells which grow depending on the presence of mouse IL-3 or TPO were cultivated in IMDM which has been supplemented with 10% heat-inactivated FCS, 0.5 mg/ml of G418 and mouse IL-3 (culture supernatant of WEHI-3B).

Using the thus cultivated Ba/F3-cmpl cells, the cell growth-stimulating activity was measured by MTT assay using the just described medium but in the absence of mouse IL-3.

The MTT assay was carried out with an inoculation density of 10,000 cells per well and by incubating the plate in 5% CO<sub>2</sub> for 48 hours.

Results of the MTT assay showed that each of TPO and TPO-ND28 (1), (2) and (3) had Ba/F3-cmp cell growth-stimulating activity.

### (3) Measurement of cell growth-stimulating activity upon NFS-60 cells

The NFS-60 cells which grow depending on the presence of human G-CSF or mouse IL-3 were cultivated in RPMI medium which has been supplemented with 10% heat-inactivated FCS, 2 mM glutamine, P/S (100 U/ml of penicillin, 100 mg/ml of streptomycin) and 1.0 ng/ml of recombinant type human G-CSF.

Using the thus cultivated NFS-60 cells, the cell growth-stimulating activity was measured by the MTT assay using the just described medium but in the absence of G-CSF.

The MTT assay was carried out with an inoculation density of 10,000 cells per well and by incubating the plate in 5% CO<sub>2</sub> for 48 hours.

Results of the MTT assay showed that each of ND28 and TPO-ND28 (1), (2) and (3) had NFS-60 cell growth-stimulating activity.

#### Test Example 3 Effect of TPO-ND28 on mouse myeloid cells

A BALB/c mouse of 8 weeks of age was sacrificed to excise the femur and tibia system whose both ends were subsequently cut with scissors. The needle of a syringe filled with RPMI solution containing 10% FCS was inserted into the section of femur and tibia to blow off myeloid cells into a small test tube, and the cells were allowed to stand for 5 minutes.

Using a Pasteur pipette, the supernatant fluid in the test tube was drawn up taking care not to contaminate it with

the precipitate, and the supernatant fluid was overlaid on Nycoprep 1.077 Animal (manufactured by NYCOMED Co., product No. 1002380) and subjected to 15 minutes of centrifugation at 600 g to isolate mouse mono nuclear cells (hereinafter referred to as "MNC").

The MNC were made into a suspension of  $5 \times 10^5$  cells/ml with a solution containing a solution to be tested, 10% FCS, 1% BSA and 0.6 mg/ml of transferrin (manufactured by Boehringer Mannheim Co.) and cultivated for 5 days in a CO<sub>2</sub> incubator (BNA-120D, manufactured by TABAI Co.) under conditions of 37°C, 5% CO<sub>2</sub> and 95% or more of humidity.

As the solution to be tested, a solution of TPO, ND28 or TPO-ND28 having a final concentration of 1.0, 10 or 100 ng/ml or a solution in which the same volume of TPO and ND-28 solutions having the above-mentioned concentration were mixed (TPO/ND28) was used. The TPO and ND28 obtained in Example 3 were used.

After completion of the cultivation, conditions of the differentiation of MNC were examined by measuring the amount of CD61 expressed which is an index of differentiation into megakaryocyte system [*J. Med.*, 311, 1084 (1984)] and the amount of Gr-1 expressed which is an index of differentiation into the granulocyte system [*J. Immunol.*, 144, 22 (1991)].

After staining with anti mouse CD61-FITC monoclonal antibody (manufactured by PHARMINGEN Co., product No. 01864D) and anti mouse Gr-1-PE monoclonal antibody (manufactured by

PHARMINGEN Co., product No. 01215A), expressed amounts of CD61 and Gr-1 were measured using an ELITE flow cytometer (manufactured by Coulter Co.).

The results are shown in Table 7.

TABLE 7

Solution to be tested	Concentration (ng/ml)	Expressed cells (%)	
		Gr-1	CD61
no addition		1.0	1.0
ND28	1.0	49.1	7.6
	10.0	40.7	4.9
	100.0	44.5	4.6
TPO	1.0	36.7	8.7
	10.0	37.7	17.8
	100.0	37.1	21.9
TPO/ND28	1.0	50.7	10.3
	10.0	40.6	10.4
	100.0	49.2	5.7
TPO-ND28	1.0	50.5	22.1
	10.0	49.8	26.6
	100.0	41.0	18.8

When the solution to be tested prepared by mixing the same amount of TPO and ND28 (TPO/ND28) was added, Gr-1 expressed cells were generated in a level similar to the case

of the addition of the solution to be tested containing ND28 alone, thus showing differentiation of MNC into the granulocyte system, but frequency of the generation of CD61 expressed cells was lower than the case of the addition of the solution to be tested containing TPO alone, thus showing decreased differentiation into the megakaryocyte system. These results suggest that, when the same amount of TPO and ND28 are present, MNC reacts mostly with ND28 and differentiates into the granulocyte system.

However, when the fusion polypeptide of TPO and ND28, namely TPO-ND28, was added as the solution to be tested, frequency of the generation of CD61 expressed cells was similar to or higher than the case of the addition of the solution to be tested containing TPO alone and two times or more higher than the case of the addition of TPO/ND28. What is more, the frequency of the generation of Gr-1 expressed cells was also similar to the case of the addition of the solution to be tested containing ND28 alone.

Test Example 4 Platelet and leukocyte production-enhancing function in mice

A 10 $\mu$ g/ml solution of TPO or a 10  $\mu$ g/ml solution of TPO-ND28 (3) obtained in Example 3 was administered by subcutaneous injection to BALB/c mice (males, 7 weeks of age) with a dose of 0.2 ml per 20 g body weight of each mouse, once a day continuously for 4 days starting on the first day of the test (treated groups, 4 animals per one group). A blood sample

was collected from the ophthalmic vein of each animal on the fifth day of the test to count the number of platelets and leukocytes by a microcell counter (Sysmex F800, manufactured by Toa Iyo Denshi Co.).

After introducing the plasmid pAGE210 used for the expression of TPO or TPO-ND28 (3) gene into CHO cells in accordance with the method described in Example 2-2), the cells were cultivated, the resulting culture supernatant was treated by the same TPO-ND28 (3) purification procedure described in Example 3, and an elution fraction corresponding to the elution fraction of TPO-ND28 (3) was used as a blank solution to count the number of platelets and leukocytes by the above-mentioned method.

In order to compare and examine effects of TPO and TPO-ND28 (3), the increasing ratio (%) of the number of platelets and leukocytes in the group in which each of these substances were administered to that in the blank solution-administered group was calculated based on the following formula:

$$\frac{[\text{platelet or leukocyte counts in mice of TPO- or TPO-ND28 (3)-administered group}]}{[\text{platelet or leukocyte count in mice of blank solution-administered group}]} \times 100$$

The results are shown in Table 8.



TABLE 8

Test substance	Increasing ratio of platelets (%)	Increasing ratio of leukocytes (%)
TPO	219	106
TPO-ND28	170	160

#### INDUSTRIAL APPLICABILITY

A fusion polypeptide comprising a polypeptide having both G-CSF activity and a polypeptide having TPO activity is provided by the present invention. The fusion polypeptide of the present invention can form and amplify platelets and leukocytes simultaneously and can control formation of megakaryocyte colonies and neutrophil colonies and differentiation or maturation of megakaryocyte precursors and neutrophil precursors.

# SEQUENCE LISTING

Sequence ID No.: 1

Sequence Length: 328

Sequence Type: amino acid

Strandedness: single

Topology: linear

Molecular Type: peptide

Original Source

Organism: human (Homo sapiens)

Sequence Characteristics:

Designation:

Location: 1..154

Designation:

Location: 154..328

Sequence

Ser	Pro	Ala	Pro	Pro	Ala	Cys	Asp	Leu	Arg	Val	Leu	Ser	Lys	Leu	Leu
1				5					10					15	
Arg	Asp	Ser	His	Val	Leu	His	Ser	Arg	Leu	Ser	Gln	Cys	Pro	Glu	Val
			20					25					30		
His	Pro	Leu	Pro	Thr	Pro	Val	Leu	Leu	Pro	Ala	Val	Asp	Phe	Ser	Leu
		35					40					45			
Gly	Glu	Trp	Lys	Thr	Gln	Met	Glu	Glu	Thr	Lys	Ala	Gln	Asp	Ile	Leu
	50					55					60				
Gly	Ala	Val	Thr	Leu	Leu	Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln
65					70					75					80

Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln  
 85 90 95  
 Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu  
 100 105 110  
 Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe  
 115 120 125  
 Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu  
 130 135 140  
 Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Thr Tyr Arg Ala  
 145 150 155 160  
 Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Ser Leu Glu Gln Val Arg  
 165 170 175  
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr  
 180 185 190  
 Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly His Ser Leu  
 195 200 205  
 Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln  
 210 215 220  
 Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln  
 225 230 235 240  
 Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr  
 245 250 255  
 Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 260 265 270  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln  
 275 280 285

Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 290 295 300  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg  
 305 310 315 320  
 Val Leu Arg His Leu Ala Gln Pro  
 325

Sequence ID No.: 2

Sequence Length: 340

Sequence Type: amino acid

Strandedness: single

Topology: linear

Molecular Type: peptide

Original Source

Organism: human (Homo sapiens)

Sequence Characteristics:

Designation:

Location: 1..153

Designation:

Location: 167..340

Sequence

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu  
 1 5 10 15  
 Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val  
 20 25 30

His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu  
 35 40 45  
 Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu  
 50 55 60  
 Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln  
 65 70 75 80  
 Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln  
 85 90 95  
 Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu  
 100 105 110  
 Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe  
 115 120 125  
 Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu  
 130 135 140  
 Val Gly Gly Ser Thr Leu Cys Val Arg Gly Gly Gly Ser Gly Gly Gly  
 145 150 155 160  
 Ser Gly Gly Gly Ser Arg Ala Pro Thr Tyr Arg Ala Ser Ser Leu Pro  
 165 170 175  
 Gln Ser Phe Leu Leu Lys Ser Leu Glu Gln Val Arg Lys Ile Gln Gly  
 180 185 190  
 Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys  
 195 200 205  
 His Pro Glu Glu Leu Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp  
 210 215 220  
 Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys  
 225 230 235 240

Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln			
	245	250	255
Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu			
	260	265	270
Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu			
	275	280	285
Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro			
	290	295	300
Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly Val Leu Val Ala			
	305	310	315
			320
Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His			
	325	330	335
Leu Ala Gln Pro			
	340		

Sequence ID No.: 3

Sequence Length: 344

Sequence Type: amino acid

Strandedness: single

Topology: linear

Molecular Type: peptide

Original Source

Organism: human (Homo sapiens)

Sequence Characteristics:

Designation:

Location: 1..153

Designation:

Location: 171..344

Sequence

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu  
1 5 10 15  
Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val  
20 25 30  
His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu  
35 40 45  
Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu  
50 55 60  
Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln  
65 70 75 80  
Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln  
85 90 95  
Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu  
100 105 110  
Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe  
115 120 125  
Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu  
130 135 140  
Val Gly Gly Ser Thr Leu Cys Val Arg Ser Gly Gly Gly Ser Gly Gly  
145 150 155 160  
Gly Ser Gly Gly Gly Ser Gly Gly Gly Arg Ala Pro Thr Tyr Arg Ala  
165 170 175

Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Ser Leu Glu Gln Val Arg  
 180 185 190  
 Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala Thr  
 195 200 205  
 Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly His Ser Leu  
 210 215 220  
 Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu Gln  
 225 230 235 240  
 Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr Gln  
 245 250 255  
 Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro Thr  
 260 265 270  
 Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile Trp  
 275 280 285  
 Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr Gln  
 290 295 300  
 Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly Gly  
 305 310 315 320  
 Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr Arg  
 325 330 335  
 Val Leu Arg His Leu Ala Gln Pro  
 340

Sequence ID No.: 4

Sequence Length: 1047

Sequence Type: nucleic acid



Strandedness: double

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Original Source

Organism: human (Homo sapiens)

Sequence Characteristics:

Designation: sig peptide

Location: 1..63

Designation: CDS

Location: 64..1047

Sequence

ATG GAG CTG ACT GAA TTG CTC CTC GTG GTC ATG CTT CTC CTA ACT GCA	48
Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu Leu Thr Ala	
-20 -15 -10	
AGG CTA ACG CTG TCC AGC CCG GCT CCT CCT GCT TGT GAC CTC CGA GTC	96
Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val	
-5 1 5 10	
CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC CTT CAC AGC AGA CTG AGC	144
Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser	
15 20 25	
CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT GTC CTG CTG CCT GCT	192
Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala	
30 35 40	

GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC CAG ATG GAG GAG ACC AAG	240
Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys	
45 50 55	
GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT CTG CTG GAG GGA GTG ATG	288
Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met	
60 65 70 75	
GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG	336
Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly	
80 85 90	
CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC	384
Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu	
95 100 105	
CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC ACA GCT CAC AAG GAT	432
Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp	
110 115 120	
CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG	480
Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val	
125 130 135	
CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC CTC TGC GTA CGG CGG GCG	528
Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala	
140 145 150 155	
CCA ACA TAT CGC GCC TCG AGT CTA CCA CAG AGC TTC CTT TTA AAA AGC	576
Pro Thr Tyr Arg Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Ser	
160 165 170	

TTA GAG CAA GTG AGG AAG ATC CAG GGC GAT GGC GCA GCG CTC CAG GAG 624  
 Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 175 180 185  
 AAG CTG TGT GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG GTG CTG 672  
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu  
 190 195 200  
 CTC GGA CAC TCT CTG GGC ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC 720  
 Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro  
 205 210 215  
 AGC CAG GCC CTG CAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC GGC 768  
 Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly  
 220 225 230 235  
 CTT TTC CTC TAC CAG GGG CTC CTG CAG GCC CTG GAA GGG ATC TCC CCC 816  
 Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro  
 240 245 250  
 GAG TTG GGT CCC ACC TTG GAC ACA CTG CAG CTG GAC GTC GCC GAC TTT 864  
 Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe  
 255 260 265  
 GCC ACC ACC ATC TGG CAG CAG ATG GAA GAA CTG GGA ATG GCC CCT GCC 912  
 Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 270 275 280  
 CTG CAG CCC ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG 960  
 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln  
 285 290 295

CGC	CGG	GCA	GGA	GGG	GTC	CTA	GTT	GCC	TCC	CAT	CTG	CAG	AGC	TTC	CTG	1008
Arg	Arg	Ala	Gly	Gly	Val	Leu	Val	Ala	Ser	His	Leu	Gln	Ser	Phe	Leu	
300					305					310					315	
GAG	GTG	TCG	TAC	CGC	GTT	CTA	CGC	CAC	CTT	GCC	CAG	CCC				1047
Glu	Val	Ser	Tyr	Arg	Val	Leu	Arg	His	Leu	Ala	Gln	Pro				
				320					325							

Sequence ID No.: 5

Sequence Length: 1083

Sequence Type: nucleic acid

Strandedness: double

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Original Source

Organism: human (Homo sapiens)

Sequence Characteristics:

Designation: sig peptide

Location: 1..63

Designation: CDS

Location: 64..1083

Sequence

ATG	GAG	CTG	ACT	GAA	TTG	CTC	CTC	GTG	GTC	ATG	CTT	CTC	CTA	ACT	GCA	48
Met	Glu	Leu	Thr	Glu	Leu	Leu	Leu	Val	Val	Met	Leu	Leu	Leu	Thr	Ala	
-20						-15					-10					

AGG CTA ACG CTG TCC AGC CCG GCT CCT CCT GCT TGT GAC CTC CGA GTC 96  
 Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val  
 -5 1 5 10  
 CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC CTT CAC AGC AGA CTG AGC 144  
 Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser  
 15 20 25  
 CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT GTC CTG CTG CCT GCT 192  
 Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala  
 30 35 40  
 GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC CAG ATG GAG GAG ACC AAG 240  
 Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys  
 45 50 55  
 GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT CTG CTG GAG GGA GTG ATG 288  
 Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met  
 60 65 70 75  
 GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG 336  
 Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly  
 80 85 90  
 CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC 384  
 Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu  
 95 100 105  
 CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC ACA GCT CAC AAG GAT 432  
 Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp  
 110 115 120

CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG	480
Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val	
125 130 135	
CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC CTC TGC GTC AGG GGT GGC	528
Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Gly Gly	
140 145 150 155	
GGT TCT GGA GGT GGT TCC GGA GGG GGT TCT AGA GCA CCA ACA TAT CGC	576
Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Arg Ala Pro Thr Tyr Arg	
160 165 170	
GCC TCG AGT CTA CCA CAG AGC TTC CTT TTA AAA AGC TTA GAG CAA GTG	624
Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Ser Leu Glu Gln Val	
175 180 185	
AGG AAG ATC CAG GGC GAT GGC GCA GCG CTC CAG GAG AAG CTG TGT GCC	672
Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys Leu Cys Ala	
190 195 200	
ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG GTG CTG CTC GGA CAC TCT	720
Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu Gly His Ser	
205 210 215	
CTG GGC ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC AGC CAG GCC CTG	768
Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser Gln Ala Leu	
220 225 230 235	
CAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC GGC CTT TTC CTC TAC	816
Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu Phe Leu Tyr	
240 245 250	

CAG GGG CTC CTG CAG GCC CTG GAA GGG ATC TCC CCC GAG TTG GGT CCC	864
Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu Leu Gly Pro	
255	260
ACC TTG GAC ACA CTG CAG CTG GAC GTC GCC GAC TTT GCC ACC ACC ATC	912
Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala Thr Thr Ile	
270	280
TGG CAG CAG ATG GAA GAA CTG GGA ATG GCC CCT GCC CTG CAG CCC ACC	960
Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu Gln Pro Thr	
285	295
CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG CGC CGG GCA GGA	1008
Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg Arg Ala Gly	
300	310
GGG GTC CTA GTT GCC TCC CAT CTG CAG AGC TTC CTG GAG GTG TCG TAC	1056
Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu Val Ser Tyr	
320	330
CGC GTT CTA CGC CAC CTT GCC CAG CCC	1083
Arg Val Leu Arg His Leu Ala Gln Pro	
335	340

Sequence ID No.: 6

Sequence Length: 1095

Sequence Type: nucleic acid

Strandedness: double

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Original Source

### Sequence Characteristics:

Location: 1..63

Designation: CDS

Location: 64..1095

## Sequence

- 72 -



GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG 336  
 Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly  
 80 85 90  
 CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC 384  
 Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu  
 95 100 105  
 CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC ACA GCT CAC AAG GAT 432  
 Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp  
 110 115 120  
 CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG 480  
 Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val  
 125 130 135  
 CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC CTC TGC GTA CGG TCC GGA 528  
 Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Ser Gly  
 140 145 150 155  
 GGT GGC TCT GGC GGT GGT TCT GGT GGC GGC TCC GGA GGC GGT CGT GCG 576  
 Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Arg Ala  
 160 165 170  
 CCA ACA TAT CGC GCC TCG AGT CTA CCA CAG AGC TTC CTT TTA AAA AGC 624  
 Pro Thr Tyr Arg Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Ser  
 175 180 185  
 TTA GAG CAA GTG AGG AAG ATC CAG GGC GAT GGC GCA GCG CTC CAG GAG 672  
 Leu Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu  
 190 195 200

AAG CTG TGT GCC ACC TAC AAG CTG TGC CAC CCC GAG GAG CTG GTG CTG 720  
 Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu  
 205 210 215  
 CTC GGA CAC TCT CTG GGC ATC CCC TGG GCT CCC CTG AGC AGC TGC CCC 768  
 Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro  
 220 225 230 235  
 AGC CAG GCC CTG CAG CTG GCA GGC TGC TTG AGC CAA CTC CAT AGC GGC 816  
 Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly  
 240 245 250  
 CTT TTC CTC TAC CAG GGG CTC CTG CAG GCC CTG GAA GGG ATC TCC CCC 864  
 Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro  
 255 260 265  
 GAG TTG GGT CCC ACC TTG GAC ACA CTG CAG CTG GAC GTC GCC GAC TTT 912  
 Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe  
 270 275 280  
 GCC ACC ACC ATC TGG CAG CAG ATG GAA GAA CTG GGA ATG GCC CCT GCC 960  
 Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala  
 285 290 295  
 CTG CAG CCC ACC CAG GGT GCC ATG CCG GCC TTC GCC TCT GCT TTC CAG 1008  
 Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln  
 300 305 310 315  
 CGC CGG GCA GGA GGG GTC CTA GTT GCC TCC CAT CTG CAG AGC TTC CTG 1056  
 Arg Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu  
 320 325 330

GAG GTG TCG TAC CGC GTT CTA CGC CAC CTT GCC CAG CCC  
Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro

1095

335

340

Sequence ID No.: 7

Sequence Length: 44

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: sig peptide

Location: 27..44

Sequence

CTCTCCAAGC TTGAATTCCG GCCAGAATGG AGCTGACTGA ATTG

44

Sequence ID No.: 8

Sequence Length: 47

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 23..47

Sequenc

GTAGAGGTAC CGCGGCCGCT TACCCTTCCT GAGACAGATT CTGGGAG

47

Sequence ID No.: 9

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

TGAACCTCTG GGCCTGGCT CAGT

24

Sequence ID No.: 10

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

GCTGCCTGCT GTGGACTTTA GCTT

24

Sequence ID No.: 11

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

TGTTGGAAGC TCAGGAAGAT GGCA

24

Sequence ID No.: 12

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

CCTGATGCTT GTAGGAGGGT CCAC

24

Sequence ID No.: 13

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

TCAAGAGTTC GTGTATCCTG TTCA

24

Sequence ID No.: 14

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

GAATGGAACT CGTGGACTCT TTCC

24

Sequence ID No.: 15

Sequence Length: 17

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence

GTAAAACGAC GGCCAGT

17

Sequence ID No.: 16

Sequence Length: 17

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence

CAGGAAACAG CTATGAC

17

Sequence ID No.: 17

Sequence Length: 13

Sequence Type: amino acid

Strandedness: single

Topology: linear

Molecular Type: peptide

Sequence

Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Arg

1

5

10

Sequence ID No.: 18

Sequence Length: 66

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..3

Designation: CDS

Location: 43..66

Sequence

TGCTCTAGAA CCGCCTCCGG AACCACTTCC AGAACCGCCA CCCCTGACGC AGAGGGTGGG 60  
CCCTCC

66

Sequence ID No.: 19

Sequence Length: 45

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA



Sequence Characteristics:

Designation: CDS

Location: 22..45

Sequence

GGTTCCGGAG GCGGTTCTAG AGCACCAACA TATCGCGCCT CGAGT

45

Sequence ID No.: 20

Sequence Length: 48

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 28..48

Sequence

CATTCCGCGG GGTACCGCGG CCGCTCAGGG CTGGGCAAGG TGGCGTAG

48

Sequence ID No.: 21

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

GGCTGCTTGA GCCAACTCCA TAGC

24

Sequence ID No.: 22

Sequence Length: 24

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..24

Sequence

GACCCAACTC GGGGGAGATC CCTT

24

Sequence ID No.: 23

Sequence Length: 57

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS

Location: 1..27

Designation: CDS

Location: 28..57

Designation: mutation

Location: 25

Designation: mutation

Location: 33..34

#### Sequence

TAGACTCGAG GCGCGATATG TTGGCGCCCG CCGTACGCAG AGGGTGGACC CTCCTAC

57

Sequence ID No.: 24

Sequence Length: 17

Sequence Type: amino acid

Strandedness: single

Topology: linear

Molecular Type: peptide

#### Sequence

Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Arg

1

5

10

15

Sequence ID No.: 25

Sequence Length: 61

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS of TPO

Location: 1..6  
Designation: linker peptide  
Location: 7..57  
Designation: CDS of ND28  
Location: 58..61  
Designation: SplI  
Location: 1..5  
Designation: MroI  
Location: 7..12  
Designation: MroI  
Location: 43..48  
Designation: BbeI  
Location: 58..61  
Designation: mutation  
Location: 4..5

#### Sequence

GTACGGTCCG GAGGTGGCTC TGGCGGTGGT TCTGGTGGCG GCTCCGGAGG CGGTCGTGCG C 61

Sequence ID No.: 26

Sequence Length: 53

Sequence Type: nucleic acid

Strandedness: single

Topology: linear

Molecular Type: other nucleic acid, synthetic DNA

Sequence Characteristics:

Designation: CDS of TPO

Location: 52..53

Designation: linker peptide

Location: 1..51

Designation: SplI

Location: 53

Designation: MroI

Location: 10..15

Designation: MroI

Location: 46..51

Sequence

ACGACCGCCT CCGGAGCCGC CACCAGAACC ACCGCCAGAG CCACCTCCGG ACC

53